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High Density Sickle Cell Erythrocyte Core Membrane Skeletons Demonstrate Slow Temperature Dependent Dissociation

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We have previously demonstrated that slow dissociation of HDSS membrane skeletons in high ionic strength Triton X-100 buffer was related to a posttranslational modification in β-actin, in which a disulfide bridge was formed between cysteine 284 and cysteine 373 [Shartava et al: J Cell Biol 128:805, 1995]. These previous dissociation assays were limited to two homozygous (SS) sickle cell patients and a single temperature (37°C). In the current work, we have expanded the SS subjects to 9 and have carried out dissociation assays at 0, 24, 30, 34, and 37°C. At 0°C there was limited dissociation of spectrin and actin from normal (AA), low density sickle cell (LDSS), and high density sickle cell (HDSS) core skeleton up to 24 hr. The first order rate constants for dissociation of spectrin, at 0°C, was 0.030–0.035 × 10^{-4}sec^{-1} for AA, LDSS, and HDSS core skeletons. However at 24, 30, 34, and 37°C the rate of dissociation of spectrin from HDSS core skeletons was significantly slower than the rate of dissociation from AA core skeletons. Having determined the first order rate constants for spectrin dissociation at these specified temperatures, we then asked whether dithiothreitol (DTT) would hasten the dissociation of core skeletons. The presence of DTT caused the rate of dissociation of the HDSS membrane skeleton to become statistically indistinguishable from the rate of dissociation of AA membrane skeletons. This is consistent with the suggestion that reversible thiol oxidation is responsible for the slow dissociation of the HDSS membrane skeleton. © 1996 Wiley-Liss, Inc.

Key words: sickle cell anemia, spectrin, actin

INTRODUCTION

There has long been substantial interest in the molecular events which occur within red blood cells (RBCs) from homozygous sickle cell (SS) patients leading to painful sickle cell crises, organ damage, and premature death [2–6]. Blood from SS patients can be separated based on density into morphologically and physiologically distinct classes of RBCs [7]. During vasoocclusion the highest density class of SS (HDSS) RBCs, which include irreversibly sickled cells (ISCs) and unsickled discocytes (USDs), are selectively trapped in the microvasculature [8–10]. ISCs appear to block the narrowed lumen of vessels, lined primarily with more adherent lower density (LDSS) reversibly sickled cells (RSCs), and directly occlude capillaries [10,11]. Therefore, as would be expected, ISCs and USDs are reduced in the peripheral blood during sickle cell crises [8,11–13].

In 1976, Lux and co-workers demonstrated that rbc membranes and Triton skeletons prepared from ISCs retained the sickled shape of the original hemoglobin containing red blood cell [14]. This observation suggested that an alteration in the membrane skeleton could be involved in the formation of the ISC. Recently we demonstrated that the slow remodelling of the ISC membrane

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skeleton is due, in part, to a posttranslational modification of β-actin in which a disulfide bridge is formed between cysteine 284 and cysteine 373 [1]. During the course of our earlier studies [1], we noticed that HDSS (enriched in ISCs) membrane skeletons appeared to dissociate more slowly at 37°C than control skeletons. These results were obtained under conditions where temperature was not perfectly regulated (water jacketed air/CO_2 incubators), and included only two SS subjects and matched controls. We felt that it was important to carry these experiments out under conditions of strict temperature control to obtain reliable rate constants, and to expand the number of SS patients tested to determine whether slow dissociation is a uniform property of HDSS membrane skeletons. The current report indicates that in nine SS subjects from eight distinct families that HDSS membrane skeletons dissociate more slowly at 24, 30, 34, and 37°C than control skeletons. The carefully obtained kinetic data described in this study has allowed us to determine the effect of a reducing agent, dithiothreitol (DTT), upon the first order rate constants for dissociation. We demonstrate that DTT causes an increased rate of dissociation of the HDSS core skeleton, while having little effect upon the rate of LDSS and control (AA) core skeletons. This is consistent with the suggestion that reversible thiol oxidation is the cause of the slow dissociation of the HDSS membrane skeleton, and therefore the slow remodelling of the ISCs which cannot change shape even when hemoglobin is depolymerized.

MATERIALS AND METHODS

Preparation of Density Separated RBCs, Ghosts, and Core Skeletons

Density separation of blood obtained from nine homozygous SS subjects from eight independent families was performed using Percoll step gradients as previously described [1]. Each density fraction within the Percoll layers was removed without cross contamination and then HDSS erythrocytes (70% Percoll), LDSS erythrocytes (45% Percoll), and control (AA) erythrocytes were washed two times in 50 ml PBS (10 mM $NaPO_4$, 150 mM NaCl, pH 7.6). Packed RBCs were lysed in 30 ml of ice cold lysis buffer (5 mM $NaPO_4$, 1 mM EDTA, pH 7.6) and ghosts were sedimented at 31,000g for 15 min at 4°C. This procedure was repeated until the pellet became white or light pink. Freshly prepared AA, HDSS, and LDSS ghosts (50 μl of packed ghosts) were incubated in 9 volumes of high ionic strength Triton X-100 buffer (10 mM $NaPO_4$, 600 mM KCl, 1 mM ATP, 1 mM DFP, and 1% Triton X-100, pH 7.6) for various times within a temperature controlled water bath. Upon completion of the extraction the samples were transferred to ice and centrifuged at 35,000g for 45 min at 4°C. The pellets were resuspended to 50 μl in lysis buffer and were analyzed by SDS-PAGE.

SDS-PAGE

HDSS, LDSS, and AA ghosts (50 μg) and the core skeletons obtained from this concentration of ghost protein were analyzed by SDS-PAGE utilizing the discontinuous buffer system of Laemmli [15] and a 9% polyacrylamide separating gel. Protein was stained with coomassie brilliant blue and scanning densitometry performed with a Zeineh laser densitometer (Biomed Instruments, Inc., Fullerton, California).

Computation and Statistical Analysis

For each temperature and various times of extraction we calculated the mean of three or more experimental values of percent of spectrin remaining in control, LDSS and HDSS core skeletons after extraction in Triton X-100 buffer. The amount of spectrin in the original ghosts (time zero) was set at 100%. First order rate constants of dissociation of spectrin was computed using the software package "Enzfitter," a non-linear regression data analysis program by Biosoft Co. (Cambridge, UK). The equation for the calculation was:

$$A = A_0 e^{-kt}$$

or

$$k = \frac{1}{t} \ln \frac{A_0}{A},$$

where A_0 is amount of protein at time zero, A is amount of protein at time t, and k is the first order rate constant of dissociation. The statistical analysis was performed using a commercially available software package SAS (Statistical Analysis System).

RESULTS

We determined the rate of dissociation of AA, HDSS, and LDSS core skeletons at 0, 24, 30, 34, and 37°C. Red blood cell membranes were isolated from these three classifications of erythrocytes and then extracted in high ionic strength Triton X-100 buffer for various times at fixed temperatures. For each temperature we present an example of the resulting SDS PAGE (Panel A) and densitometric analysis of spectrin remaining in the skeleton (Panel B). In Table I we present the mean ± standard error, for three to six independent experiments at each temperature, of the first order rate constants for spectrin's dissociation from the core skeleton.

At 0°C extraction of AA, LDSS, and HDSS erythrocytes in high ionic strength Triton X-100 buffer led to

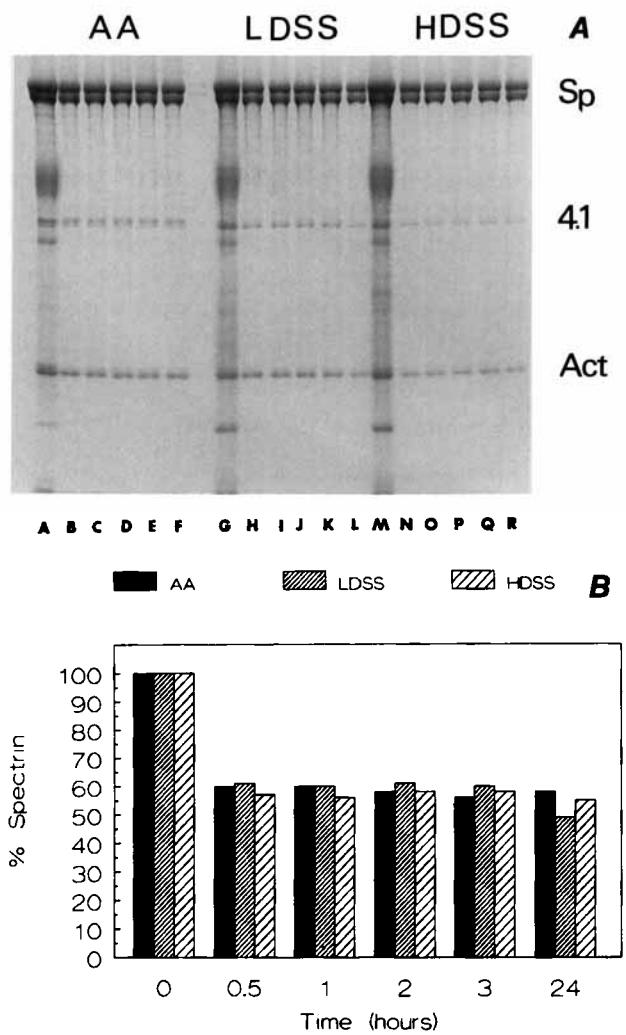

Fig. 1. Core Membrane Skeleton Dissociation at 0°C. A: SDS PAGE of 50 μg of AA (A), LDSS (G), and HDSS (M) membrane protein; and core skeletons isolated from 50 μg of AA (B–F), LDSS (H–L), and HDSS (N–R) membranes. The skeletons were prepared by extraction in high ionic strength Triton X-100 buffer at 0°C for 0.5 hr (B, H, N), 1 hr (C, I, O), 2 hr (D, J, P), 3 hr (E, K, Q), and 24 hr (F, L, R). B: Densitometric analysis of the amount of spectrin remaining in the core skeletons at various times of extraction at 0°C. The amount of spectrin in the original ghosts (time zero) was set at 100%.

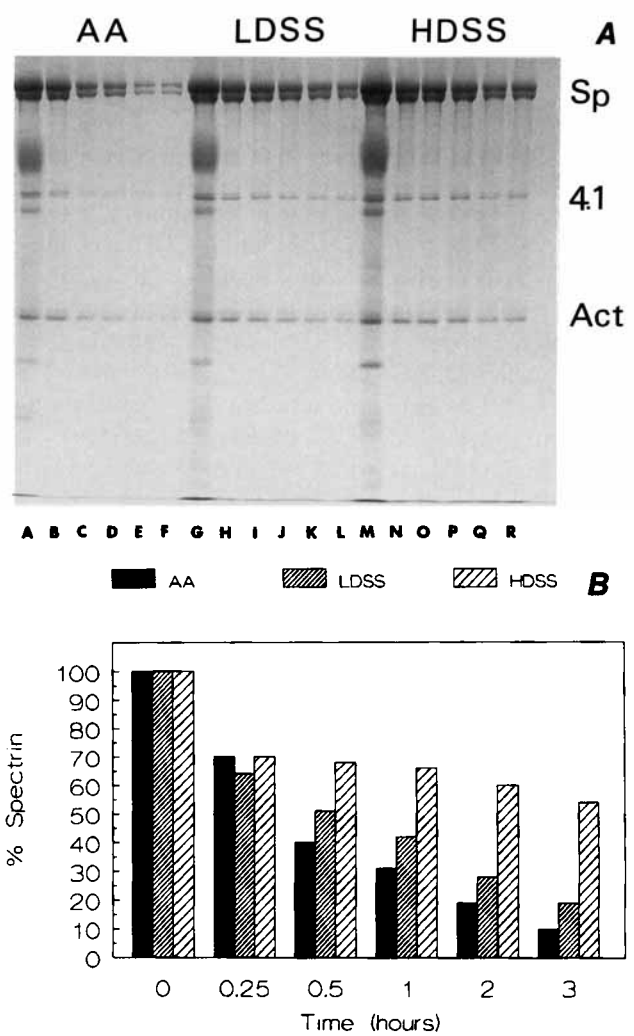

Fig. 2. Core Membrane Skeleton Dissociation at 24°C. A: SDS-PAGE of 50 μg at AA (A), LDSS (G), and HDSS (M) membrane protein, and core skeletons isolated from 50 μg of AA (B–F), LDSS (H–L), and HDSS (N–R) membranes. The skeletons were prepared by extraction in high ionic strength Triton X-100 buffer at 24°C for 15 min (B, H, N), 30 min (C, I, O), 1 h (D, T, P), 2 h (E, K, Q), and 3 h (F, L, R). B: Densitometric analysis of the amount of spectrin remaining in the core skeletons at various times of extraction at 24°C. The amount of spectrin in the original ghosts (time zero) was set at 100%.

TABLE I. First Order Rate Constants ($10^{-4}sec^{-1}$) of Dissociation of Membrane Skeletons at Different Temperatures†

	Dissociation temperature				
Membrane	0°C	24°C	30°C	34°C	37°C
AA	0.035 ± 0.033 (4)	3.78 ± 0.88 (3)	11.15 ± 0.79 (6)	47.50 ± 4.50 (3)	71.33 ± 12.33 (3)
LDSS	0.035 ± 0.032 (4)	1.75 ± 0.50 (3)	9.72 ± 0.98 (6)	40.50 ± 1.50 (3)	56.17 ± 10.67 (3)
HDSS	0.030 ± 0.022 (4)	0.83 ± 0.35 (3)*	7.29 ± 0.99 (6)*	20.67 ± 5.00 (3)*	40.17 ± 6.83 (3)*
AA + DTT			11.01 ± 0.77 (3)		
LDSS + DTT			11.05 ± 0.97 (3)		
HDSS + DTT			9.78 ± 0.56 (3)		

†Mean ± standard error.

*Denotes rate constants which are significantly different from the control (AA) values ($P < 0.05$).

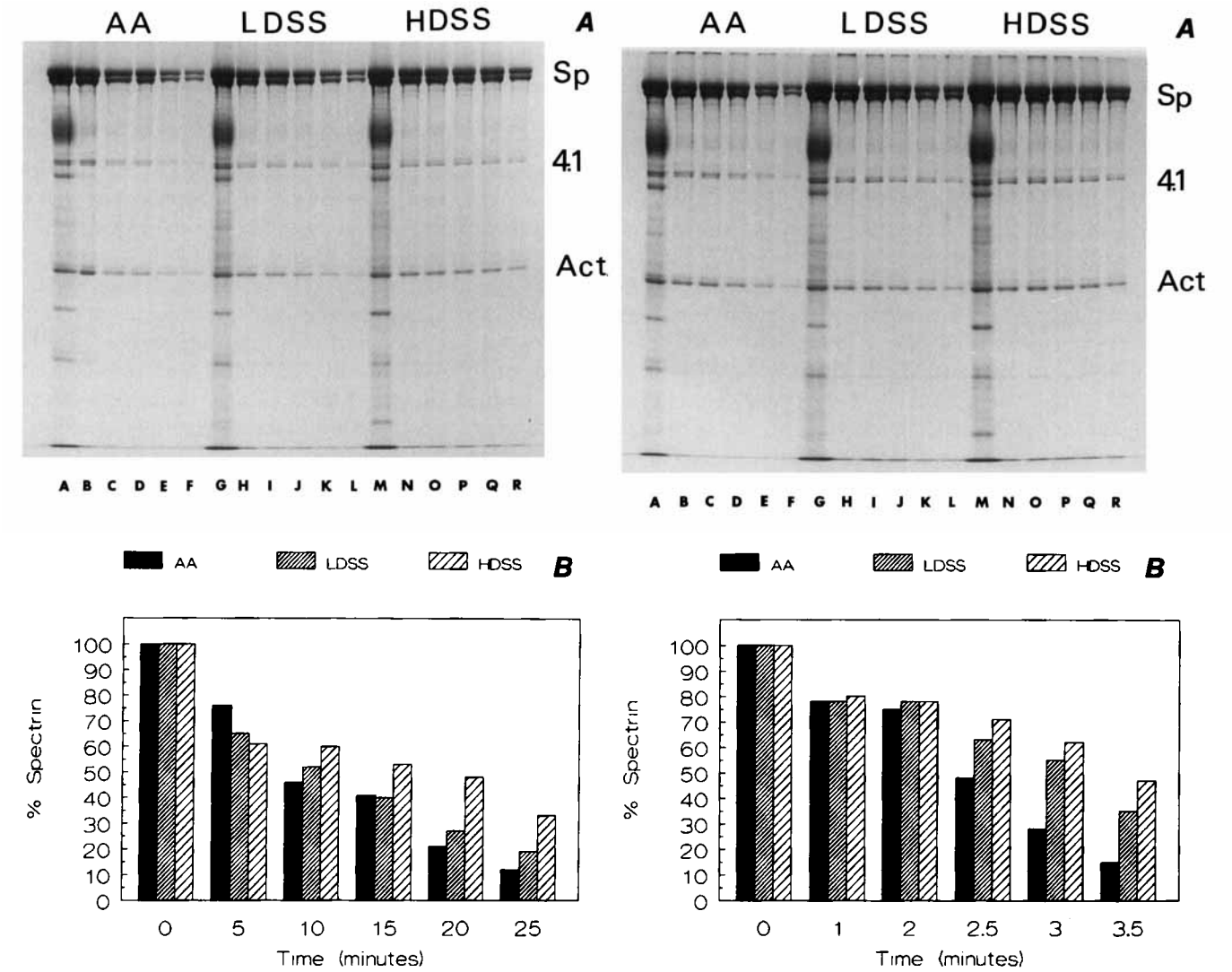

Fig. 3. Core Membrane Skeleton Dissociation at 30°C. A: SDS-PAGE of 50 μg AA (A), LDSS (G), and HDSS (M) membrane proteins, and core skeletons isolated from 50 μg of AA (B–F), LDSS (H–L), and HDSS (N–R) membranes. The skeletons were prepared by extraction in high ionic strength Triton X-100 buffer at 30°C for 5 min (B, H, N), 10 min (C, I, O) 15 min (D, T, P), 20 min (E, K, Q), and 25 min (F, L, R). B: Densitometric analysis of the amount of spectrin remaining in the core skeletons at various times of extraction at 30°C. The amount of spectrin in the original ghosts (time zero) was set at 100%.

Fig. 4. Core Membrane Skeleton Dissociation at 37°C. A: SDS-PAGE of 50 μg AA (A), LDSS (G), and HDSS (M) membrane proteins, and core skeletons isolated from 50 μg of AA (B–F), LDSS (H–L), and HDSS (N–R) membranes. The skeletons were prepared by extraction in high ionic strength Triton X-100 buffer at 37°C for 1 min (B, H, N), 2 min (C, I, O) 2.5 min (D, T, P), 3 min (E, K, Q), and 3.5 min (F, L, R). B: Densitometric analysis of the amount of spectrin remaining in the core skeletons at various times of extraction at 37°C. The amount of spectrin in the original ghosts (time zero) was set at 100%.

core skeletons containing primarily spectrin, protein 4.1, and actin (Fig. 1A). The densitometric analysis of the SDS-PAGE shown in Figure 1A is presented in Fig. 1B. In three independent experiments on blood obtained from three SS subjects and controls, 60–80% of spectrin remains associated with the core skeletons up to 24 hr of extraction. The first order rate constants, at 0°C, for the dissociation of spectrin were the same for AA, LDSS, and HDSS core skeletons within the error of the measurement ($0.030–0.035 \times 10^{-4}sec^{-1}$) (Table I).

At 24°C, over a 3 hr time course, we observe greater dissociation of AA core skeletons than HDSS core skeletons (Fig. 2A,B); with intermediate values for LDSS skeletons. The first order rate constants were $3.78 \pm 0.33 \times 10^{-4}sec^{-1}$ (AA), $1.75 \pm 0.50 \times 10^{-4}sec^{-1}$ (LDSS), and $0.83 \pm 0.35 \times 10^{-4}sec^{-1}$ (HDSS) for spectrin dissociation from the core skeleton. Therefore the rate of dissociation is 4–5 times slower for the HDSS skeleton as compared to the AA skeleton at 24°C, and

the differences are statistically significant ($P < 0.05$) (Table I).

At 30°C (Fig. 3A,B) the first order rate constants for spectrin dissociation from the core skeleton are $11.15 \pm 0.79 \times 10^{-4}sec^{-1}$ (AA), $9.72 \pm 0.98 \times 10^{-4}sec^{-1}$ (LDSS), and $7.29 \pm 0.99 \times 10^{-4}sec^{-1}$ (HDSS). At 30°C the rate of dissociation was ~35% slower for HDSS, vs. AA, core skeletons. Again the differences between the dissociation rate constants for HDSS and AA membrane skeleton are significant ($P < 0.05$) (Table I).

Extraction at 34°C (data not shown) yielded first order rate constants (Table I) of $47.50 \pm 7.50 \times 10^{-4}sec^{-1}$ (AA), $40.50 \pm 7.50 \times 10^{-4}sec^{-1}$ (LDSS), and $20.67 \pm 5.00 \times 10^{-4}sec^{-1}$ (HDSS). At 34°C the rate of dissociation was again 56% slower for HDSS, vs. AA, core skeletons, and was statistically significant ($P < 0.05$) (Table I).

When extraction was performed at 37°C (Fig. 4A,B) the first order rate constants were $71.33 \pm 12.33 \times 10^{-4}sec^{-1}$ (AA), $56.17 \pm 10.67 \times 10^{-4}sec^{-1}$ (LDSS), and $40.17 \pm 6.83 \times 10^{-4}sec^{-1}$ (HDSS) for spectrin dissociation (Table I). Once again the rate of dissociation was ~44% times slower for the HDSS core skeletons, as compared to the AA core skeletons. The differences in rate constant for HDSS and AA core skeletons was significant ($P < 0.05$) (Table I).

With the first order rate constants, for spectrin dissociation from the core skeletons, well defined; we decided to ask whether the reducing agent DTT would effect the rate of dissociation. We prepared ghosts, as described in the Materials and Methods section, ±1 mM DTT in the lysis buffer, and then carried out the high ionic strength Triton X-100 extraction ±5 mM DTT at 30°C. This temperature was selected because close to complete dissociation occurs for the control skeletons within 25 min in the absence of DTT while ~30% of the HDSS skeletons remain intact. While DTT had no statistically significant effect upon the rate of dissociation of spectrin from AA and LDSS core skeletons (Table I), it increased the first order rate constant for HDSS core skeleton to a value, $9.78 \pm 0.56 \times 10^{-4}sec^{-1}$, which was no longer statistically distinct from the AA rate constants (see Table I).

DISCUSSION

In the current study we have demonstrated that, at all temperatures tested between 24 and 37°C, spectrin dissociates more slowly from HDSS core skeletons than from AA or LDSS core skeletons. These studies were carried out under more rigorous temperature control (water bath) than our previous observations (air/CO_2 incubator) [1]. We also expanded the patient population tested and studied the dissociation kinetics at several temperatures (in addition to 37°C). The conclusion remains that HDSS skeletons dissociate more slowly, than AA and LDSS core skeletons and this may explain the slow remodelling of the ISC membrane skeleton.

The assay for membrane skeleton dissociation in high ionic strength Triton X-100 buffer, established in the current study, should be of value in the testing of drugs to hasten the dissociation rate of HDSS, or ISC, core skeletons in vitro. Our previous studies demonstrated that a disulfide bridge between cysteine 284 and cysteine 373 in β-actin is the major cause of the slow dissociation of the ISC membrane skeleton (1) suggesting that membrane permeable reducing agents may be of value. In our recent studies we have demonstrated that dithiothreitol (DTT) can block the formation of ISCs in vitro, and can convert ISCs formed in vivo back to RSCs (Campbell et al., manuscript in preparation). In the current study we demonstrate that DTT can hasten the dissociation of the HDSS or ISC core skeleton; demonstrating the value of this assay in testing the efficacy of various drugs on membrane skeletal interactions. The rate parameters, described in the current article, will allow the use of this assay to test various nontoxic reducing agents and antioxidants to help determine the most promising therapeutic agents for blocking ISC formation in patients with sickle cell anemia.

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REFERENCES

1. Shartava A, Monteiro CA, Benscath FA, Schneider K, Chait B, Gussio R, Cassoria-Scott LA, Shah AK, Heuerman CA, Goodman SR: A postranslational modification of β-actin leads to persistently sickled membrane skeleton of irreversibly sickled cells. J Cell Biol 128:805, 1995.
2. Hebbel RP: The sickle erythrocyte in double jeopardy: Autoxidation and iron decompartmentalization. Sem Hematol 27:51, 1990.
3. Hebbel RP: Beyond hemoglobin polymerization: The red blood cell membrane and sickle disease pathophysiology. Blood 77:214, 1991.
4. Powers DR: Sickle cell anemia and major organ failure. Hemoglobin 14:573, 1990.
5. Francis RB Jr, Johnson CS: Vascular occlusion in sickle cell disease: Current concept and unanswered questions. Blood 77:1405, 1991.
6. Joiner CH: Cation transport and volume regulation in sickle red blood cells. Am J Physiol 264:C251, 1993.
7. Fabry ME, Benjamin L, Lawrence C, Nagel RL: An objective sign in painful crisis in sickle cell anemia: The concomitant reduction in high density red cells. Blood 79:1602, 1984.
8. Kaul DK, Fabry ME, Windisch P, Baez S, Nagel RL: Erythrocytes in sickle cell anemia in their rheological and hemodynamic characteristics. J Clin Invest 72:22, 1983.
9. Kaul DK, Fabry ME, Nagel RL: Vaso-occlusion by sickle cells: evidence for selective trapping of dense red cells. Blood 68:1162, 1986.

10. Kaul DK, Fabry ME, Nagel RL: Microvascular sites and characteristics of sickle cells adhesion to vascular endothelium in shear flow conditions: Pathophysiological implications. Proc Natl Acad Sci USA 86:3356, 1989.
11. Ballas SK, Larner T, Smith ED, Surrey S, Schwartz E, Rappaport EF: Reologic predictors of the severity of the painful sickle cell crisis. Blood 72, 1216, 1988.
12. Lande WM, Andrews DL, Clark MR, Braham NV, Black DM, Embury SH: Crisis in homozygous sickle cell disease: Correlation with red cell deformability. Blood 72:2056, 1988.
13. Ballas SK, Smith ED: Red blood cell changes during the evolution of the sickle cell painful crisis. Blood 79:2154, 1992.
14. Lux SE, John KM, Karmovsky MT: Irreversible deformation of the spectrin-actin lattice in irreversibly sickled cells. J Clin Invest 58:995, 1976.
15. Laemmli, UK: Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature (Lond.) 227:680–685, 1970.